Published in final edited form as:

Transplant Proc. 2010 ; 42(6): 2052–2054. doi:10.1016/j.transproceed.2010.05.118.

Tissue Dissociation Enzyme Neutral Protease Assessment

A.G. Breite, F.E. Dwulet, and R.C. McCarthy

VitaCyte LLC, Indianapolis, Indiana, USA

Abstract

Neutral proteases, essential components of purified tissue dissociation enzymes required for successful human islet isolation, show variable activities and effects of substrate on their activities. Initially we used a spectrophotometric endpoint assay with azocasein substrate to measure neutral protease activity. After critical review of the results, we observed these data to be inconsistent and not correlating expected differences in specific activities between thermolysin and *Bacillus polymyxa* proteases. This observation led to the development of a fluorescent microplate assay using fluorescein isothiocyanate-conjugated bovine serum albumin (FITC-BSA) as the substrate. This simpler, more flexible method offered a homogeneous, kinetic enzyme assay allowing determination of steady state reaction rates of sample replicates at various dilutions. The assay had a linear range of 4- to 8-fold and interassay coefficients of variation for *B. polymyxa* protease and thermolysin of <9% and <15%, respectively, which were lower than those using the spectrophotometric endpoint assay, namely, 54% and 36%, respectively. This format allowed for incorporation of enzyme inhibitors, as illustrated by addition of sulfhydryl protease inhibitors, which, consistent with earlier reports, strongly indicated that the main contaminant in purified collagenase preparations was clostripain. Determination of the specific activities for several purified neutral proteases showed that the *B. polymyxa* and *Clostridium histolyticum* proteases had approximately 40% and 15% specific activities, respectively, of those obtained with purified thermolysin, indicating the different characteristics of neutral protease enzymes for cell isolation procedures.

Collagenase and neutral protease are essential components of tissue dissociation enzymes (TDE) that are required to release islets and other cells^{1–4} from extracellular matrices. The focus of many reports has been on the characteristics and performance of collagenases in islet isolation.⁵ A recent report by Brandhorst et al showed that enzyme mixtures containing purified neutral protease and collagenase from *Clostridium histolyticum* gave higher human islet yields when using selected lots of collagenase that contained greater amounts of trypsin-like activity (TLA).⁶ This observation stresses the importance of neutral protease in islet isolation and the need to further characterize the assays that are used to measure this activity.

Historically, neutral protease assays are usually endpoint methods that use natural or derivatized proteins as the substrate—casein or bovine serum albumin (BSA)—instead of peptide substrates.^{7–10} The major limitation of using these substrates is that many procedures use time-consuming precipitation with trichloroacetic acid (TCA) to separate undigested proteins from soluble peptides.^{8,9} In our hands, the performance of an endpoint

Address reprint requests to Andrew G. Breite, VitaCyte LLC, 1102 Indiana Avenue, Indianapolis, IN 46202, USA.
abreite@vitacyte.com.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

neutral protease assay using an azocasein substrate was problematic, because of excessive variability in the blank samples, low signal to noise ratios leading to high inter-assay coefficients of variation (typically >20%), and inconsistent performance when comparing the specific activities of certain enzymes when compared with the other assay substrates.

The disadvantages of traditional neutral protease assays led to the development of a fluorescent microplate assay that used fluorescein isothiocyanate (FITC)-conjugated BSA (FITC-BSA) as the substrate.¹¹ We used the same assay format as previously described for a collagen degradation assay.¹² This report summarized the characteristics and use of this assay to determine the specific activities of 3 neutral proteases for islet isolation procedures, illustrating an application using specific inhibitors which confirmed that clostripain is a common contaminant of purified collagenase products.

METHODS

Thermolysin and *Bacillus polymyxa* protease were purified by anion-exchange chromatography. Collagenases from *C. histolyticum* culture supernates were purified by a sequential chromatographic separation procedure that resulted in separations of Class II (C2) from Class I (C1) collagenase.¹³ *C. histolyticum* neutral protease was obtained as an enriched pool by modifications of the affinity purification process of Sparrow and McQuade.¹⁴

A spectrophotometric endpoint neutral protease assay using azocasein as substrate was performed as described by Sarath et al.¹⁰ Neutral protease activity (NPA) was measured as azocasein units (AUC), which were defined as the amount of enzyme required to release an A₄₄₀ equivalent to 1 mg/mL of azocasein per mL per minute in a reaction at 37°C. The fluorescence microplate assay used the same format as described earlier for the fluorescent collagen degradation assay.¹² FITC-BSA final concentration of 100 µg/mL was used as the substrate. The conjugate was prepared using a modified protocol.¹¹ NPA was expressed as neutral protease units (NPU), which were defined as an increase of 1 fluorescent unit per minute at 35°C. Specific NPAs were calculated by dividing the enzyme unit (ACU or NPU) by the micrograms of protein per well, assuming 1 mg of purified CI or CII shows an A280 of 1.41, 1 mg of purified thermolysin had 1.1, and purified *B. polymyxa* protease had 1.0. Analytical anion exchange high-performance liquid chromatography (HPLC) and (SDS-PAGE) were performed as described previously.

RESULTS

The specific NPAs for purified thermolysin and *B. polymyxa* protease (ie, equivalent to Dispace) showed similar specific activities (15 U/mg) using azocasein as the substrate. In contrast, specific NPA using the fluorescent microplate assay showed specific NPAs of 90,000 and 260,000 NPU/mg protein for *B. polymyxa* protease and thermolysin, respectively. This 2.9-fold difference in the specific NPA was consistent with earlier observations, showing differences in turnover number between these enzymes (F.E. Dwulet, unpublished observation). When compared with the endpoint assay the fluorescent microplate assay displayed improved precision to assess NPA. The between-run coefficient of variation (CV) for the specific NPAs of *B. polymyxa* protease and thermolysin were 54% and 37%, respectively, using the endpoint assay. In contrast, using the fluorescent microplate assay the specific NPAs showed between-run CVs of 8.2% and 14.8% for *B. polymyxa* protease and thermolysin, respectively.

The fluorescent microplate assay was also used to characterize the proteases that contaminate purified collagenase preparations. The activity was not inhibited by aspartyl-, metallo-, or serine protease inhibitors, but was strongly blocked by several sulfhydryl

protease inhibitors (Fig 1). The strong inhibition by *N*-ethylmaleimide (NEM) indicated that the contaminant was likely clostripain, a common contaminant of purified collagenase that has been shown to be responsible for TLA.¹⁵ In a separate experiment >98% of clostripain NPA was inhibited by NEM. NEM had minimal impact on other neutral proteases, including thermolysin and *B. polymyxa* protease (data not shown). NEM inhibition of this NPA may serve as another measurement of TLA in purified collagenase products.

C. histolyticum neutral protease (CHNP) shows similar preferences for specific amino acid sequences as found for thermolysin and *B. polymyxa* protease, which cleave on the amino terminal side of hydrophobic amino acids such as leucine.^{16–18} However, little is known about how this enzyme compares with other neutral proteases used in islet isolation. An 80% enriched pool of this enzyme, as determined using coomassie-stained SDS-PAGE, showed a predominant band at 34 kD (Fig 2), which is similar to the 33 kD estimate reported earlier in the patent literature.¹⁹ The specific activity of the enriched CHNP pool was approximately 30,000 NP U/mg using the FITC-BSA substrate. This observation indicates that the specific NPA of this CHNP preparation was about 33% of *B. polymyxa* protease (90,000 NPU/mg) and 11% of thermolysin (260,000 NPU/mg), illustrating the differences in turnover number of proteases used in islet isolation procedures.

DISCUSSION

Neutral protease is known to play a critical role in the dissociation of pancreatic tissue for islet release, although its exact role is not completely understood because of the complex proteinaceous structure of the extracellular matrix.²⁰ All current neutral protease assays that use enriched or purified proteins as substrates are poor surrogate to assess enzyme activities that are required for effective islet isolation. However, some type of NPA assay is required for the manufacture and quality characterization of these enzymes to achieve a more consistent product for cell isolation procedures.

The fluorescent microplate assay described above has several benefits when compared with traditional endpoint assays, which are often used to characterize these enzymes. It is a homogeneous assay, meaning that there is no separation step required to measure substrate degradation. The increased relative fluorescence measured during the course of the assay reflects the release of small fluorescent peptides into the assay mixture. The use of a microplate format enables repeated measurements during the assay from a larger number of samples leading to a more accurate assessment of the kinetic activity of the enzyme. This advantage enables improved characterization of these enzymes by assessing the effect of specific inhibitors on their activity. And lastly, the interassay CVs were <9% and <15% for *B. polymyxa* protease and thermolysin, respectively, which were significantly lower than those observed using traditional endpoint neutral protease assays.

This assay provides an improvement over the assays used today by manufacturers of TDE products,^{7,9} but additional developments must be performed to better characterize NPA. This endeavor may require replacement of purified protein substrates with more complex proteins that better approximate the extracellular matrix. The value of using more complex substrates will be determined only after the results of this assay and traditional neutral protease methods are compared for prediction of the yields of enzyme activity on islets and other cells.

Acknowledgments

Supported by grants from the National Institutes of Health (NIH). VitaCyte supported by NIH grants R43DK065467, R43DK070402, and R43DK079413 and NIH SBIR Match Awards and a 21st Century Research and Technology Award from the State of Indiana.

References

1. Hatton MW, Berry LR, Krestynski F, et al. The role of proteolytic enzymes derived from crude bacterial collagenase in the liberation of hepatocytes from rat liver. Identification of two cell-liberating mechanisms. *Eur J Biochem* 1983;137:311. [PubMed: 6317390]
2. Hefley TJ, Stern PH, Brand JS. Enzymatic isolation of cells from neonatal calvaria using two purified enzymes from *Clostridium histolyticum*. *Experimental Cell Research* 1983;149:227. [PubMed: 6315459]
3. McShane P, Sutton R, Gray DW, et al. Protease activity in pancreatic islet isolation by enzymatic digestion. *Diabetes* 1989;38 (suppl 1):126. [PubMed: 2642834]
4. Suggs W, Van Wart H, Sharefkin JB. Enzymatic harvesting of adult human saphenous vein endothelial cells use of a chemically defined combination of two purified enzymes to attain viable cell yields equal to those attained by crude bacterial collagenase preparations. *J Vasc Surg* 1992;15:205. [PubMed: 1309465]
5. Kin T, Johnson PRV, Shapiro AMJ, et al. Factors influencing the collagenase digestions phase of human islet isolation. *Transplantation* 2007;83:7. [PubMed: 17220782]
6. Brandhorst H, Friberg A, Andersson HH, et al. The importance of tryptic-like activity in purified enzyme blends for efficient islet isolation. *Transplantation* 2009;87:370. [PubMed: 19202441]
7. Lin Y, Means GE, Feeney RE. The action of proteolytic enzymes on *N, N*-dimethyl proteins. *J Biol Chem* 1969;244:789. [PubMed: 4889948]
8. Matsubara H. Thermolysin. *Methods Enzymology* 1970;19:642.
9. Twining SS. Fluorescein isothiocyanate-labeled casein assay for proteolytic enzymes. *Analytical Biochem* 1984;143:30.
10. Sarath, G.; De La Motte, R.; Wagner, FW. *Proteolytic Enzymes, A Practical Approach*. New York: IRL Press; 1989. Protease assay methods; p. 25
11. Voss EW, Workman CJ, Mummert ME. Detection of protease activity using fluorescence-enhancement globular substrate. *BioTechniques* 1996;20:286. [PubMed: 8825159]
12. McCarthy RC, Spurlin B, Wright MJ, et al. Development and characterization of a collagen degradation assay to assess purified collagenase used in islet isolation. *Transplant Proc* 2008;40:339. [PubMed: 18374061]
13. Hefley TJ. Utilization of FPLC-purified bacterial collagenase for the isolation of cells from bone. *J Bone Min Res* 1987;2:505.
14. Sparrow LG, McQuade AB. Isolation by affinity chromatography of neutral proteinase from *Clostridium histolyticum*. *Bio-chimica et Biophysica Acta* 1973;302:90.
15. Mitchell WM. The contamination of purified collagenase preparations by clostridiopeptidase B (clostripain): the potential effect on studies utilizing collagenase as a highly specific structural tool. *John Hopkins Medical* 127:192, 197.
16. McQuade AB, Crewther WG. Peptide substrates for a proteinase of *Clostridium histolyticum*. *Biochimica et Biophysica Acta* 1968;167:619. [PubMed: 5722284]
17. Morihara K, Tsuzuki H. Comparative study of various neutral proteinases from microorganisms: specificity with oligopeptides. *Arch of Biochem & Biophys* 1971;146:291. [PubMed: 5004124]
18. Griffin PJ, Fogarty WM. Physicochemical properties of the native, zinc- and manganese- prepared metalloprotease of *Bacillus polymyxa*. *Appl Microbiol* 1973;26:191. [PubMed: 4743873]
19. Hesse, F.; Ambrosius, D.; Bartscher, H. Recombinant proteinase from *Clostridium histolyticum* and its use for isolating cells and groups of cells. United States Patent 1998. p. 853-976.
20. Martinez-Hernandez A. Electron microscopy of the extracellular matrix: an overview. *Meth Enzymol* 1987;145:78. [PubMed: 3600400]

Fig 1.

Effect of inhibitor on residual NPA in a purified collagenase blend. TLA attributed to the sulfhydryl protease clostripain by >90% inhibition of protease activity by sulfhydryl protease inhibitors *N*-ethylmaleimide, Pefabloc SC, and Leupeptin. *N*-ethylmaleimide is specific for cysteine residues while Pefabloc SC and Leupeptin are general serine and cysteine proteases inhibitors indicating that clostripain is the likely source of TLA activity in purified collagenase preparations.

Fig 2. Coomassie-stained SDS-PAGE gel image of *C. histolyticum* neutral protease-enriched pool after affinity chromatography purification. Lane 1 is Pierce 3 Color Prestained molecular weight marker with bands from top to bottom at approximate molecular weights of 210 kd, 110 kd, 80 kd, 47 kd, 32 kd, and 25 kd. Lanes 2 and 3 are 2 separately prepared enriched pools of *C. histolyticum* neutral protease.